BT0102

DSP Rapid Kit 48 Ver.2009.4.27

A colorimetric phosphatase inhibition assay

(DSP: Diarrhoeic Shellfish Poisoning)

Introduction

Diarrhoeic Shellfish Poisoning (DSP) is caused by the ingestion of shellfish contaminated by toxic dinoflagellates. DSP has been observed worldwide and caused problems to public health and the shellfish industry. To detect DSP toxins, a colorimetric phosphatase inhibition assay was developed using a highly purified recombinant human PP2A C-subunit. The assay is very sensitive, fast, easy, accurate and reproducible to detect DSP toxins (OA group) in the shellfish. It does not require a high level of skills. The lowest quantifiable limit of OA is 0.1 μg/g in shellfish whole meat.

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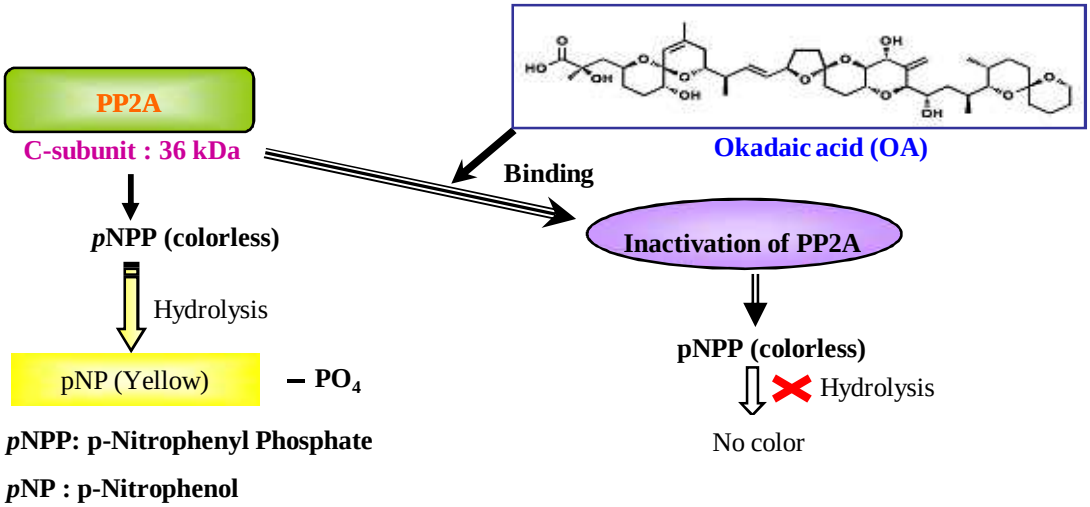
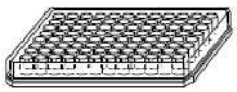
Assay Principle

The assay is based on the inhibition of the protein phosphatase (PP2A) by DSP toxins (OA and DTXs). PP2A can hydrolyze a colorless artificial substrate, *p*-nitrophenyl phosphate (*p*-NPP), and produces the yellow color of *p*-nitrophenol (*p*-NP) in the alkaline solution. The intensity of the color is proportional to the enzyme activity and the absorbance is measured at 405 nm. The concentration of DSP toxins in the sample is calculated from the standard curve produced using known concentrations of OA.



Summary of Assay



plate

← **Add 50 µl** of **sample** and **OA Reference Solution**

← **Add 100 µl** of **PP2A Working Solution**

← **Add 100 µl** of **Substrate Working Solution**

Mix for 1 min by a microplate mixer

↓

Incubate for 30 min at 36℃

↓

Measure absorbance at 405 nm against 490 or 492nm as reference

Kit Components

Item		Amount	Storage
1	Okadaic Acid (OA) Reference Solutions (OA R1~OA R8)	8 x 0.5 ml	-20℃
2	Sample Buffer	20 ml	-20℃
3	1.25 N NaOH Solution	1.5 ml	-20℃
4	1.25 N HCl Solution	1.5 ml	-20℃
5	PP2A Buffer A	5.5 ml	-20℃
6	PP2A Buffer B	0.5 ml	-20℃
7	PP2A Stock Solution (0.5 ml tube)	40 μl	-20℃
8	Substrate Buffer	12 ml	-20℃
9	Substrate Tablet (*p*-NPP)	20 mg tablet	-20℃
10	1×8 wells×6 strips		
11	Adhesive Film	1	
12	containing Excel spread sheet*		

***The Excel spreadsheet is on the website(http://www.ttc.co.jp/dsp/).**

Caution: This kit contains strong alkali and acid solutions, and toxin (Okadaic acid). Wear disposable gloves, eye protection and protective clothing when preparing and handling reagents and samples.

The PP2A Stock solution of this kit might contain a small amount of recombinant Baculovirus because of its manufacturing method.

Additional Materials Required

- Microplate reader capable of measuring absorbance at 405 nm against 405 or 492nm as reference
- Microplate mixer
- Incubator for use at 36℃
- Heating apparatus: water bath or heating block
- Vortex mixer
- Reagent reservoirs
- Micro pipettes (20-200 ml and 200-1000 ml) with disposable tips.
- Multichannel pipette
- 15 ml and 50 ml Polyethylene tubes
- 1.5 ml Microcentrifuge tubes
- 2 ml Screw capped tubes
- Deionized or distilled water

Sample Preparation

Sample for Free OAs (OA, DTX1, and DTX2)

Collect the whole meat of shellfish.

↓ Remove excess water by placing on filter papers.

Mince the whole meat and weigh 2 g into a 50 ml centrifuge tube.

↓ ← Add 18 ml of 90% methanol. (9 times of weighed minced sample)

Homogenize for 1 min with Polytron homogenizer or an equivalent (any other capable homogenizer) at room temperature.

↓ Centrifuge at 2,500×g for 10min. at room temperature.

Transfer the middle part of supernatant to a suitable tube.
(the supernatant is designated **Extract Supernatant**)

↓ Keep the Extract Supernatant at room temperature.

Pipette 50 µl of Extract Supernatant into a 1.5 ml microcentrifuge tube and add 950 µl of the Sample Buffer **(Sample for Free OAs)**.

Note: Sample Buffer may contain precipitates that can be dissolved by shaking at room temperature.
Extract supernatant storage : store at -20 ℃
Extract supernatant return to the room temperature and dissolve precipitation before using. (Do NOT heat !)

Sample for Total OAs (OA, DTX1, DTX2 and DTX3)

Pipette 500 µl of the **Extract Supernatant** into a screw-capped 2 ml tube.

↓ Add 100 µl of 1.25 N NaOH solution, tighten the cap and mix.

Heat at 100℃ for 20 min or at 80℃ for 40 min.

↓

Cool the tube to room temperature and add 100 µl of 1.25 N HCl to neutralize the solution.

↓

Pipette 50 µl of the neutralized solution into a 1.5ml microcentrifuge tube and add 950 µl of the Sample Buffer **(Sample for Total OAs)**.

Preparation of Working Solution

Before preparing Substrate Working Solution and PP2A Working Solution, defrost OA Reference Solutions, PP2A Buffer A, PP2A Buffer B and Substrate Buffer (Kit Components #: 1, 5, 6 and 8).

Keep OA Reference Solutions (#1) at room temperature, and keep the other solutions (#5, 6, and 8) at 4℃ or on ice.

PP2A Stock Solution (#7) should be taken out from -20 ℃ right before the PP2A Working Solution preparation and kept at 4℃ or on ice.

1. Substrate Working Solution

Substrate buffer + Substrate Tablet (*p*-NPP)

Dissolve the Substrate tablet in Substrate Buffer. Wrap the tube with aluminium foil and keep at 4℃ or on ice.

2. PP2A Working Solution

PP2A Buffer A + PP2A Buffer B + PP2A stock solution

First, spin down PP2A Buffer B and PP2A Stock Solution (#6 and 7).

Add PP2A Buffer B (#6; 0.5ml) to PP2A Buffer A (#5; 5.5ml) and mix well (invert slowly!).

Then, add PP2A Stock Solution (#7; 35µl) to the solution (Buffer A and B mixed) and mix well (invert slowly!).

The PP2A Working Solution should be kept at 4℃ or on ice and should be used on that day.

Assay Procedure

An example for placing OA Reference Solutions and test samples in the 48-well (1 x 8 wells x 6strips) microplate is shown below.

TRIPLICATES of OA Reference Solutions and samples are recommended for the higher reliability of data.

	1	2	3	4	5	6	7	8	9	10	11	12
A	OA R1	OA R1	OA R1	SF1	SF1	SF1						
B	OA R2	OA R2	OA R2	SF2	SF2	SF2						
C	OA R3	OA R3	OA R3	SF3	SF3	SF3						
D	OA R4	OA R4	OA R4	SF4	SF4	SF4						
E	OA R5	OA R5	OA R5	ST1	ST1	ST1						
F	OA R6	OA R6	OA R6	ST2	ST2	ST2						
G	OA R7	OA R7	OA R7	ST3	ST3	ST3						
H	OA R8	OA R8	OA R8	ST4	ST4	ST4						

Example

SF: Sample for Free OAs **ST: Sample for Total OAs**

1. Add 50 µl of the OA Reference Solutions and Samples to each well.

2. Add 100 µl of PP2A Working Solution. A multichannel pipette is recommended to use .

3. Add 100 µl of Substrate Working Solution. A multichannel pipette is recommended to use.

4. Seal the plate with the Adhesive Film and mix for 1 minute with a microplate mixer.

5. **Incubate the plate for 30 minutes at 36℃** and get the microplate reader ready.

6. After incubation, carefully remove the Adhesive Film and measure absorbance with a microplate reader at 405 nm against 490 or 492 nm as reference.

Quality Assurance

Quality of the performed assay is assured by the following criteria.

1. The absorbance value for OA-R1 should reach 0.4 or over.
2. The absorbance value for OA-R8 should be smaller than 0.15.
3. The relative error** must be smaller than 10%, if a possible outlier is excluded from the three run data in the assay.
   **(data A – data B)/(data A + data B), where data A > data B.

Data Calculation

The data calculation is performed semi-automatically on a Microsoft Excel spreadsheet.* (* cf. page 3)

1. Input the net-absorbance values obtained for OA Reference Solutions into cells colored light blue in the Table placed at the top of the sheet.
2. Check the relative standard deviation values. If the value exceeds 10%, a possible outlier should be excluded.
3. The standard curves for the net-absorbance values and percent of residual enzyme activity are drawn at the middle left and right of the sheet, respectively.
4. Input the net-absorbance values obtained for the sample solutions to the corresponding cells which are tabulated at the bottom of the sheet and colored light green. The sample numbers with prime denote hydrolyzed samples.
5. Check the relative standard deviation values. If the value exceeds 10%, a possible outlier should be excluded by erasing the cell for the outlier.
6. The calculation results expressed in terms of OA-equivalents are shown in the right end column in μg/g. Values less than 0.1 μg (OA-equivalent)/g tissues are expressed as <0.1 μg/g. For calculation of the total OA-equivalent, a factor “1.4” is used to calibrate the value increase due to added alkali and acid solutions.

References

1. Ikehara T, Shinjo F, Ikehara S, Imamura S, Yasumoto T.: Baculovirus expression, purification, and characterization of human protein phosphatase 2A catalytic subunits alpha and beta. Protein Expr Purif. 45(1):150-6, 2006.
2. Tubaro A, Florio C, Luxich E, Sosa S, Della Loggia R, Yasumoto T.: A protein phosphatase 2A inhibition assay for a fast and sensitive assessment of okadaic acid contamination in mussels. Toxicon 34(7):743-52, 1996.
3. Takai A, Mieskes G.: Inhibitory effect of okadaic acid on the p-nitrophenyl phosphate phosphatase activity of protein phosphatases. Biochem J. 275:233-9, 1991

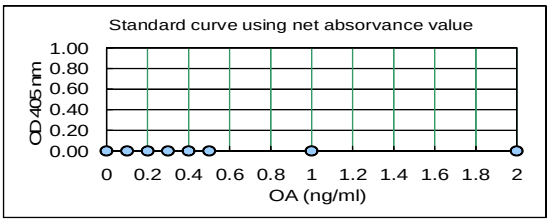
Example of Data Calculation Sheet

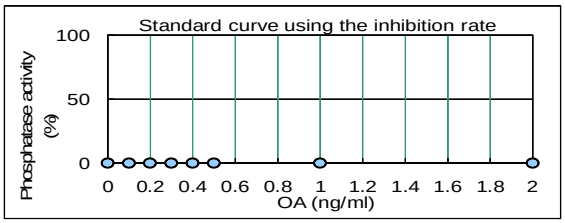
DATE 2008/ / ( )
Laboratory:

＜Data calculation sheet＞

Net absorbance values

OA reference solutions	R1	R2	R3	R4	R5	R6	R7	R8
OA concentration (ng/ml)	0	0.1	0.2	0.3	0.4	0.5	1	2
run 1								
run 2								
run 3								
Average	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
relative standard deviation	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
Phosphatase activity (%)	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!





sample name	Hydrolysis*1	run 1	run 2	run 3	average	relative standard deviation(%)	result (μg/g)*2
1					#DIV/0!	#DIV/0!	#DIV/0!
2					#DIV/0!	#DIV/0!	#DIV/0!
3					#DIV/0!	#DIV/0!	#DIV/0!
4					#DIV/0!	#DIV/0!	#DIV/0!
5					#DIV/0!	#DIV/0!	#DIV/0!
6					#DIV/0!	#DIV/0!	#DIV/0!
1'	h				#DIV/0!	#DIV/0!	#DIV/0!
2'	h				#DIV/0!	#DIV/0!	#DIV/0!
3'	h				#DIV/0!	#DIV/0!	#DIV/0!
4'	h				#DIV/0!	#DIV/0!	#DIV/0!
5'	h				#DIV/0!	#DIV/0!	#DIV/0!
6'	h				#DIV/0!	#DIV/0!	#DIV/0!

*1 For hydrolysis sample input "h" in the column indicated "hydrolysis" to enable automatically calibrate the result.
*2 The result is expressed as the concentration in the sample in μg/g.

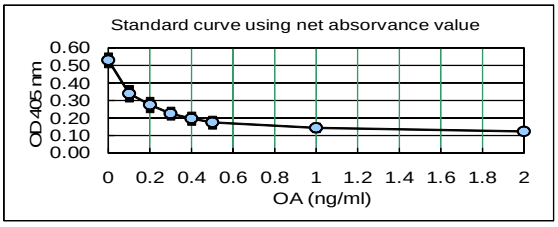
Calculation Example

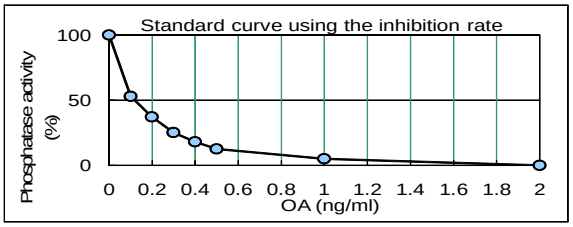
DATE 2007/10/15 (Mon)
Laboratory: TTC

＜Data calculation sheet＞

Net absorbance values

OA reference solutions	R1	R2	R3	R4	R5	R6	R7	R8
OA concentration (ng/ml)	0	0.1	0.2	0.3	0.4	0.5	1	2
run 1	0.549	0.351	0.279	0.232	0.203	0.180	0.147	0.124
run 2	0.522	0.327	0.265	0.221	0.194	0.172	0.143	0.124
run 3	0.520	0.339	0.281	0.225	0.194	0.173	0.143	0.123
Average	0.5303	0.3390	0.2750	0.2260	0.1970	0.1750	0.1443	0.1237
relative standard deviation	3.1%	3.5%	3.2%	2.5%	2.6%	2.5%	1.6%	0.5%
Phosphatase activity (%)	100.0	53.0	37.2	25.2	18.0	12.6	5.1	0.0





sample name	Hydrolysis*1	run 1	run 2	run 3	average	relative standard deviation(%)	result (μg/g)*2
1					#DIV/0!	#DIV/0!	#DIV/0!
2					#DIV/0!	#DIV/0!	#DIV/0!
3					#DIV/0!	#DIV/0!	#DIV/0!
4					#DIV/0!	#DIV/0!	#DIV/0!
5					#DIV/0!	#DIV/0!	#DIV/0!
6					#DIV/0!	#DIV/0!	#DIV/0!
1'	h				#DIV/0!	#DIV/0!	#DIV/0!
2'	h				#DIV/0!	#DIV/0!	#DIV/0!
3'	h				#DIV/0!	#DIV/0!	#DIV/0!
4'	h				#DIV/0!	#DIV/0!	#DIV/0!
5'	h				#DIV/0!	#DIV/0!	#DIV/0!
6'	h				#DIV/0!	#DIV/0!	#DIV/0!

*1 For hydrolysis sample input "h" in the column indicated "hydrolysis" to enable automatically calibrate the result.
*2 The result is expressed as the concentration in the sample in μg/g.